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14. ABSTRACT Pathways that mediate the ubiquitin dependent degradation of androgen receptor (AR) remain to be established, and it is apparent that ubiquitylation regulates AR functions in addition to the degradation of unliganded AR. We hypothesize that there are distinct ubiquitin ligases and pathways regulating the degradation and transcriptional functions of liganded nuclear AR versus the unliganded cytoplasmic AR. One of our major objectives is to identify such pathways mediating the turnover of liganded nuclear AR, as these may be exploited therapeutically through the development of selective AR antagonists. Our approach has been to use initially mass spectrometry and identify AR sites that undergo ubiquitylation in the presence and absence of androgen. We have now identified a series of such sites, and current efforts are focused on determining the functional significance of these sites.

15. SUBJECT TERMS
Prostate cancer, Androgen receptor, ubiquitin, degradation

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INTRODUCTION

The ability of prostate cancer (PCa) cells to restore androgen receptor (AR) function despite treatment with CYP17A1 inhibitors or novel AR antagonists has stimulated interest in the development of AR antagonists or other agents that can drive AR degradation. However, pathways that normally mediate the ubiquitin dependent or independent degradation of AR, which might be targeted therapeutically, remain to be established. The rate of AR degradation is increased substantially in the absence of androgen, and this degradation is proteasome dependent (Sheflin et al., 2000). This increased degradation is likely preceded by AR polyubiquitylation, but the ubiquitylation of specific sites on AR that drive this process and the relevant ubiquitin ligases remain to be firmly established. CHIP (C-terminus of Hsc70 interacting protein) is a ubiquitin ligase that interacts with the molecular chaperones HSP90 and Hsc70, and links this molecular chaperone system to the ubiquitin-proteasome system. CHIP may mediate the polyubiquitylation of the unliganded AR, possibly through an interaction with the AR N-terminus (He et al., 2004), but the role of CHIP in AR degradation remains to be clarified (Adachi et al., 2007; Cardozo et al., 2003; Morishima et al., 2008; Rees et al., 2006).

AR also may be ubiquitylated by other ubiquitin ligases (including MDM2, UBCH7, TAF1, RNF6, TRIM68, and PIRH2), although many of these may mediate monoubiquitylation and/or function to modulate (enhance or suppress) the transcriptional activity of the liganded AR (Chymkowitch et al., 2010; Gaughan et al., 2005; Lin et al., 2002; Tavassoli et al., 2010; Xu et al., 2009). Ubiquitin ligases also may recognize distinct structural features of the liganded AR, such as AKT mediated phosphorylation of S213 or CDK7 mediated phosphorylation of S515, which have been reported to stimulate MDM2 binding (Chymkowitch et al., 2010; Lin et al., 2002), versus the HSP90 associated unliganded AR that may be targeted for degradation due to exposure of hydrophobic surfaces. Therefore, it is apparent that ubiquitylation regulates AR functions in addition to the degradation of unliganded AR, and we hypothesize that there are distinct ubiquitin ligases and pathways regulating the degradation of liganded nuclear AR versus the unliganded AR associated with HSP90. One of our major objectives is to identify such pathways mediating the turnover and degradation of liganded nuclear AR, as these may be exploited therapeutically through the development of selective AR antagonists that activate these pathways, in analogy with fulvestrant in breast cancer which enhances the degradation of nuclear ERα.

BODY

Aim 1. Identify and characterize ubiquitylation sites on AR

Subaim 1a. Identify ubiquitylation sites associated with degradation of the unliganded AR. We have used liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) to identify ubiquitylation sites on AR in cells cultured in androgen depleted medium versus medium with androgen (see subaim 1b). For these studies we focused on VCaP cells, as they have an amplified wild-type AR, and thereby express high levels of AR protein. In a representative experiment we immunoprecipitated AR from cell lysates of multiple large plates of VCaP cells cultured in steroid-depleted medium and treated with a proteasome inhibitor. The AR was then run on SDS-PAGE and a region running above the unmodified AR was excised from the gel (as well as the ~110 kD unmodified AR band in another gel slice). Protein in gel slices was then digested with trypsin (which leaves a gly-gly tag on ubiquitin-modified proteins)

and analyzed by LC-MS/MS.

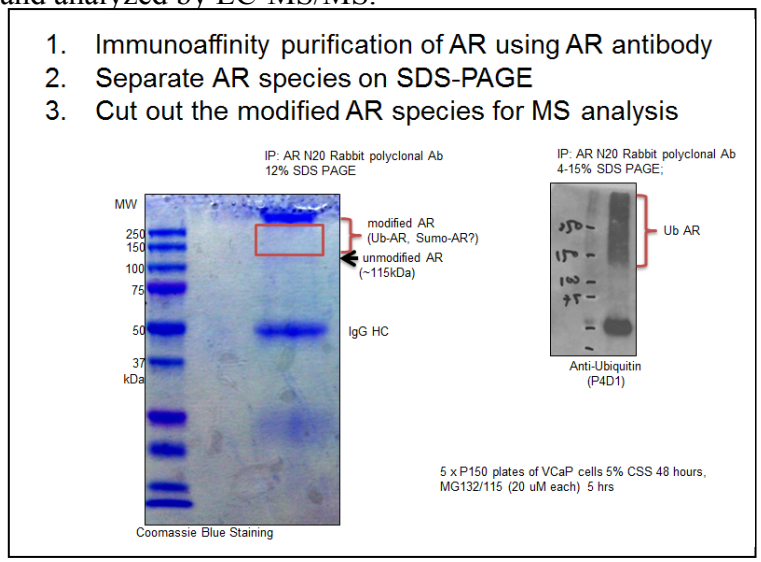

Figure 1. Identification of the ubiquitylation sites of AR using mass spectrometry (LC-MS/MS). AR from VCaP cells cultured in steroid-depleted medium with MG132/115 was immunopurified, run on SDS-PAGR and areas corresponding to ubiquitylated or intact AR were excised. Right panel show an anti-ubiquitin blot of the immunopurified AR.

Figure 2 shows the LC-MS/MS profile from one experiment, and a list of the post-translational modifications in AR that were detected in a series of 3 similar preparations and analyses.

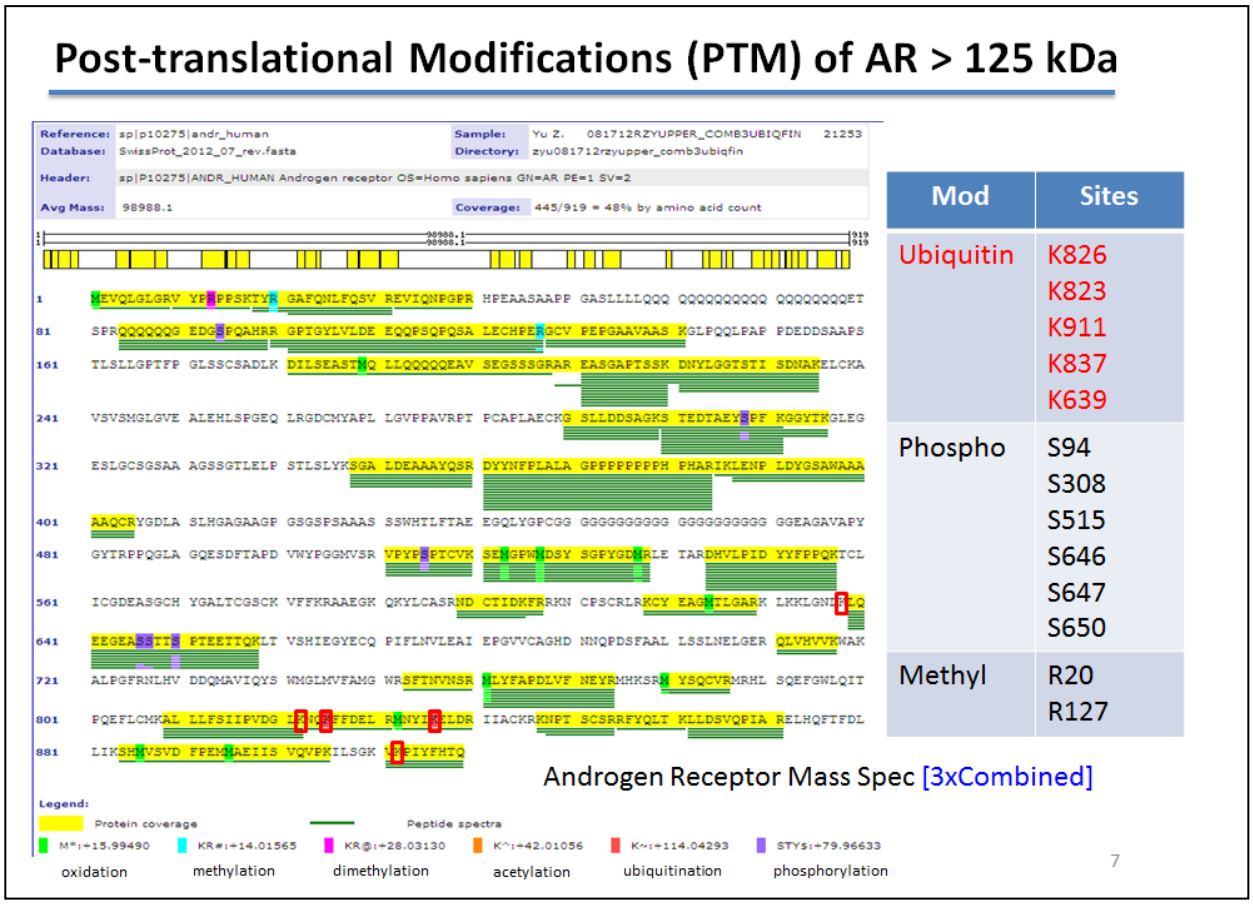

Figure 2. Posttranslational modifications of AR in androgen-depleted VCaP cells.

It should be noted that peptide coverage was limited in each study, and that there are areas of the AR that we have not been able to detect by LC-MS/MS despite using additional enzymes for cleavage. Therefore, we cannot rule out additional ubiquitylation sites. Indeed, sites at 630 and/or 632 in the hinge region, and 845 and 847 in the ligand binding domain have been reported.

Subaim 1b. Identify ubiquitylation sites associated with the liganded AR. We have carried out similar analyses from androgen (DHT) stimulated VCaP cells. The combined results from a series of LC-MS/MS analyses of DHT stimulated or depleted cells is shown in figure 3.

Condition	Appox.MW (kDa)	Ubiquitylation	Phosphorylation	Methylation	Acetylation
No DHT + MG132	>125	K639 K826 ~~K823~~ K911 K837	S94 S308* S515* S646 S647 S650*	R20 R127 K658	
	~115	K911 ?	S94* S119 S256 S308 S515 S529 Y530 S646 S647 S650*	K311 K316 K633	K826
+ DHT + MG132	>125	K718 K862 K911	S256 S308 S650	R20 R127	
	~115		S308 S521 T649 S650	K639	K220 K311

Figure 3. Summary of AR post-translational modifications in DHT and androgen-depleted VCaP cells detected by LC-MS/MS. Lysines that are circled had more than one modification.

Subaim 1c. Determine the functional significance of ubiquitylation sites. To identify functions for these sites, we have used site directed mutagenesis to convert each lysine to arginine. To further establish that specific sites undergo ubiquitylation, we cotransfected cells with HA epitope tagged ubiquitin and wild-type or mutant ARs. We then immunoprecipitated AR and immunoblotted for HA-ubiquitin. As shown in figure 4, mutation of K837 or K911 alone did not decrease ubiquitylation, but the double mutant had markedly decreased ubiquitylation. Similar studies are underway for the other sites and are being extended to other cells including stably transfected PC3 and LNCaP cells, but this result supports K837 and K911 as major ubiquitylation sites.

To assess for functions in regulating AR protein degradation, we used lentivirus to express V5-tagged wildtype or mutant ARs in LNCaP cells. AR protein stability in androgen depleted cell was then assessed by treating with cycloheximide to block new protein synthesis, and examining AR protein over a time course. The results indicate that ubiquitylation at 639, 837, and 911 can contribute to AR degradation under low androgen conditions (Figure 5). We have

further examined whether K837 or K911 contribute to the AR degradation in response to blocking HSP90 with geldanamycin. In this experiment, C4-2 cells were transiently transfected with Flag-tagged wild-type of mutant ARs and then treated with a proteasome inhibitor (MG132, MG), geldanamycin (GA), or the combination. As expected, geldanamycin treatment resulted in decreased AR protein, and this could be restored by proteasome inhibition (Figure 6). In contrast, both the K837R and K911R mutations prevented the geldanamycin mediated decrease in AR protein. These findings suggest that ubiquitylation at both sites is required for the enhanced AR degradation observed after HSP90 inhibition.

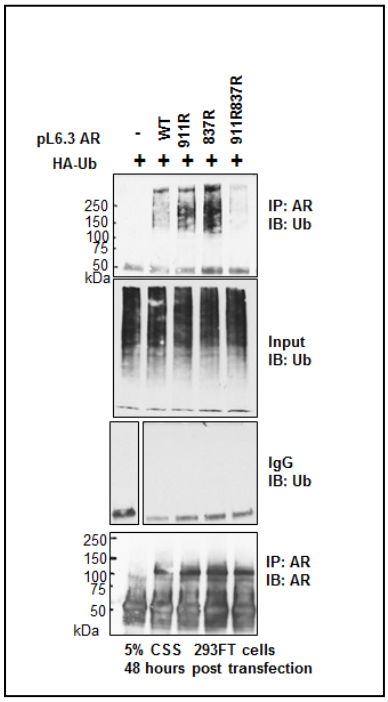

Figure 4 (left). Ubiquitylation of wild-type (WT) versus mutant ARs. 293T cells were transfected as indicated for 3d in steroid-depleted medium. Lysates were then immunoprecipitated with anti-AR or control IgG, and then blotted with anti-HA to detect ubiquitin (top and 3rd panel) or with anti-AR (bottom panel). Input is blotted for HA (2nd panel)

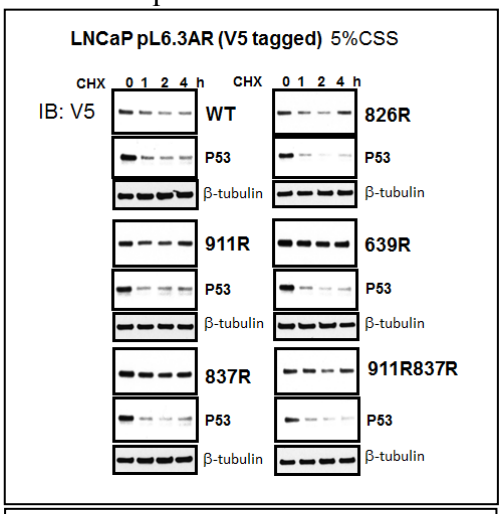

Figure 5. Ubiquitylation sites contribute to AR degradation. LNCaP cells were transduced with indicated ARs. Cells in steroid-depleted medium were then treated with cycloheximide for 0-4 hours.

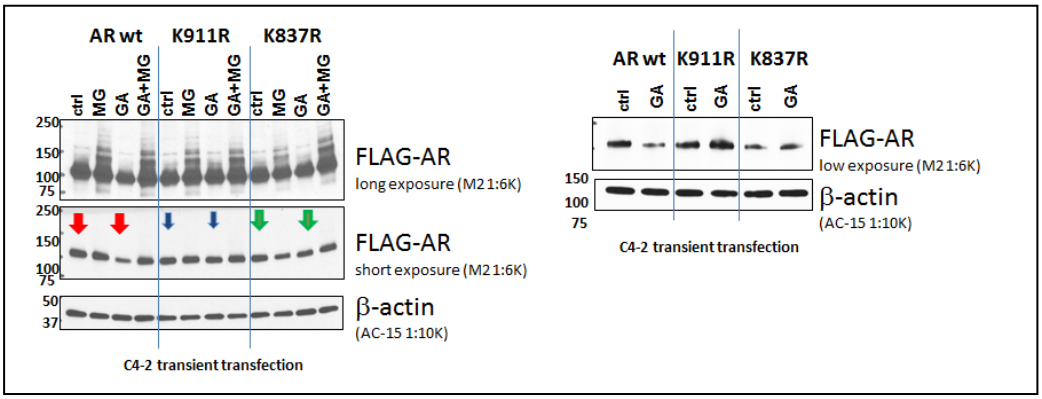

Figure 6. K911 and K837 mediate geldanamycin-induced AR degradation. Left panel, C4-2 cells were transiently transfected with WT or mutant AR expression vectors. After 2 days they were treated with proteasome inhibitor (MG132, MG), geldanamycin (GA), or the combination for 6 hours. They were then harvested and lysates were blotted for AR. Right panel, repeat experiment.

Aim 2. Identify ubiquitin dependent mechanisms mediating nuclear and cytoplasmic AR degradation

a. Identify ubiquitin ligases mediating AR polyubiquitylation and degradation. We obtained expression vectors for a series of E3 ubiquitin ligases that have been implicated in AR regulation, as well as additional ones expressed in prostate cancer cells. These were transiently transfected with AR into PC3 cells, and AR protein was assessed by immunoblotting. As expected, both SKP2 and MDM2 decreased AR protein (Figure 7). In addition, FBL and TRCP E3 ligases decreased AR protein. We are currently assessing effects of these E3 ligases on the panel of lysine mutant ARs.

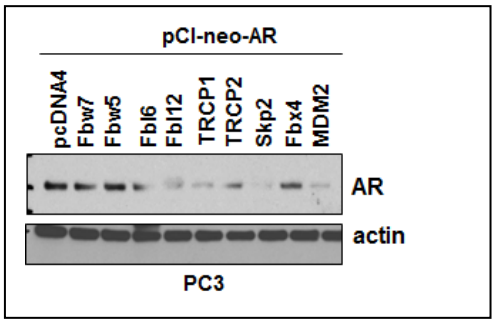

Figure 7. Effects of E3 ligases on AR protein expression. PC3 cells were transiently transfected with wild-type AR and the indicated E3 ligases. AR protein levels were then assessed by immunoblotting after 3 days.

We have also taken advantage of available deubiquitinase (DUB) inhibitors to determine whether AR degradation may be prevented by one or more DUBs that remove ubiquitin. LNCaP cells cultured in steroid-depleted medium were treated with the indicated DUB inhibitors, alone or with a proteasome inhibitor (MG132) for overnight. Effects on AR protein were then determined by immunoblotting (Figure 8). We also blotted for p53 as a positive control that undergoes rapid MDM2 dependent degradation that can be rescued by MG132. The results indicate that AR may be stabilized by one or more DUBs, and indicate that UCH-L1 is one such DUB. These studies are currently being extended to the mutant ARs to identify specific sites that may be involved.

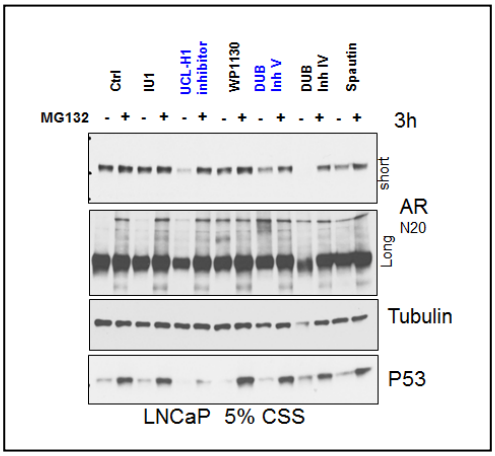

Figure 8. Effects of DUB inhibitors on AR protein. LNCaP cells in steroid-depleted medium were treated overnight with the indicated DUBs, alone or in combination with MG132. The DUBs targeted by the inhibitors are as follows:
IU1: usp14 inhibitor
UCH-L1 inhibitor: inhibits the oncogenic DUB UCH-L1 (ubiquitin c-term hydrolase L1)
WP1130: partially selective DUB inhibitor (USP14, UCH-L1, UCH37 etc), inhibits JAK/STAT signaling.
DUB inhibitor V: broad DUBs inhibtor
DUB inhibitor IV: usp14, UCH37/UCH-L5 inhibitor
Spautin: inhibits usp10 and 13

b. Identify AR degradation pathways that may be enhanced by AR antagonists. We recently reported that the AR antagonist galeterone could enhance degradation of the T878A mutant AR (Yu et al., 2014). Studies are pending to assess whether this is mediated through a specific pathway and site.

KEY RESEARCH ACCOMPLISHMENTS

-identification of sites on AR that are ubiquitylated under steroid-depleted conditions

-identification of sites on AR that are ubiquitylated in the presence of androgen
-generation and preliminary characterization of panel of ARs with mutations at each ubiquitylation site

REPORTABLE OUTCOMES

We reported that a novel AR antagonists that is now in clinical trials, galeterone, could markedly increase degradation of the T878A mutant AR (Yu et al., 2014).

CONCLUSIONS

AR undergoes ubiquitylation at a series of sites that have not been identified or characterized previously. Our initial studies suggest that K911 and K837 are major sites, but it appears that the other sites may also contribute to AR degradation. Further studies that are underway will establish the functional significance of each site and identify the pathways leading to their ubiquitylation.

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